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Properties of copper-free pig kidney amine oxidase: Role of topa quinone

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Abstract Copper removal from pig kidney amine oxidase containing Cu/topaquinone (TPQ) has been obtained using CN^- in the presence of the poor substrate *p*-(dimethylamino)benzylamine. Upon removal of copper, the enzyme loses its activity while the TPQ cofactor remains in its oxidized form. The addition of copper to the apo-form fully restores the active enzyme. The CN^- treatment in the presence of sodium dithionite or good substrates (cadaverine or benzylamine) also removes copper but the TPQ cofactor is irreversibly reduced and the addition of copper does not regenerate the active enzyme. Ni^{II} and Zn^{II} do not bind the apo-protein in contrast to Co^{II} which is incorporated to the same extent as Cu^{II}. However, Co-reconstituted enzyme only shows a very low activity. These results demonstrate that copper is essential for the catalytic mechanism because it maintains the correct active site geometry.

Keywords: Amine oxidase; Pig kidney; Cofactor; Copper; 6-Hydroxydopa

1. Introduction

The superfamily of amine oxidases (AOs) isolated from mammals, many higher-order plants, fungi and bacteria is formed by a group of enzymes involved in cellular and extracellular metabolism of amines [1]. AOs have been divided into two main categories depending on the cofactor involved and on the catalytic mechanism. One class is characterized by the presence of flavin adenine dinucleotide (FAD) as the redox cofactor. The enzymes belonging to this class are further subdivided into monoamine oxidases (MAO A and MAO B) and polyamine oxidases (PAOs) depending on the substrate specificity.

The second class is represented by enzymes possessing tightly bound Cu^{II} and a quinone cofactor. These enzymes can be subdivided into two subclasses based on the type of quinone cofactor present: 6-hydroxydopa quinone (TPQ or TOPA) derived from the oxidation, in a post-translational event, of a tyrosinyl residue in the amino acid sequence [2,3], or lysyl tyrosylquinone (LTQ) derived from the crosslinking of the ε-amino group of a lysine and a modified tyrosine within the same polypeptide chain [4].

The class of Cu/quinone amine oxidases includes:

(i) The TPQ-containing intracellular AOs, also called diamine oxidases because of their preference for diamines as substrate.
(ii) The TPQ-containing intracellular surface associated AOs, often indicated as semicarbazide-sensitive amine oxidases.
(iii) The TPQ-containing mammalian circulating AOs, termed either plasma or serum AOs, or benzylamine oxidases because of their "in vitro" preferential activity towards this non-physiological substrate.
(iv) The LTQ-containing extracellular matrix-bound lysyl oxidase that catalyzes maturation and aging of collagen and elastin through the oxidation of lysyl residues in these fibrous proteins.

Copper/TPQ-containing amine oxidases [amine:oxygen oxidoreductase (deaminating) EC 1.4.3.6] are homodimers, each subunit (molecular mass 70–90 kDa) containing a tightly bound copper ion and a TPQ. These enzymes catalyze the oxidative deamination of mono-, di- and polyamines, abstracting two electrons from the amino groups and transferring them to molecular oxygen, yielding the corresponding aldehyde, ammonia and hydrogen peroxide. The catalytic mechanism can be divided in two half-reactions: (1) enzyme reduction at the quinone moiety (TPQ → $TPQH_2$) by substrate, followed by (2) its reoxidation by molecular oxygen:

$$E_{ox} + \text{R-CH}_2\text{-NH}_3^+ \rightarrow E_{red} + \text{R-CHO} \quad (1)$$

$$E_{red} + O_2 + H_2O \rightarrow E_{ox} + NH_4^+ + H_2O_2. \quad (2)$$

The AO from pig kidney (PKAO) is one of the best known and its catalytic mechanism, detailed in Scheme 1, has been previously reported [5]. The species A–D of the reductive half-reaction (Eq. (1) and Scheme 1) are very similar in AOs from different sources [4,6]. On the other hand, the oxidative half-reaction (Eq. (2)) implicates two still controversial points: the formation of Cu^I-semiquinolamine cation radical species (Scheme 1E; TPQ_{sq}) and the role of copper. In plant AOs

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Abbreviations: AO, amine oxidase; Cu_{dep}, Cu-fully-depleted; Cu_{rec}, Cu-fully-reconstituted; *p*-DABA, *p*-(dimethylamino)benzylamine; PHY, phenylhydrazine; PKAO, pig kidney amine oxidase; TPQ, 6-hydroxydopa (2,4,5-trihydroxyphenylalanine) quinone; TPQ_{aqu}, aminoquinol (the reduced form of TPQ after treatment with *p*-DABA); TPQ_{aqu2}, aminoquinol (the reduced form of TPQ after treatment with cadaverine or benzylamine); TPQ_{thb}, trihydroxybenzene, TPQ reduced by dithionite; TPQ_{ox}, TPQ in the oxidized form; TPQ_{sq}, TPQ semiquinone (Cu^I-semiquinolamine radical)

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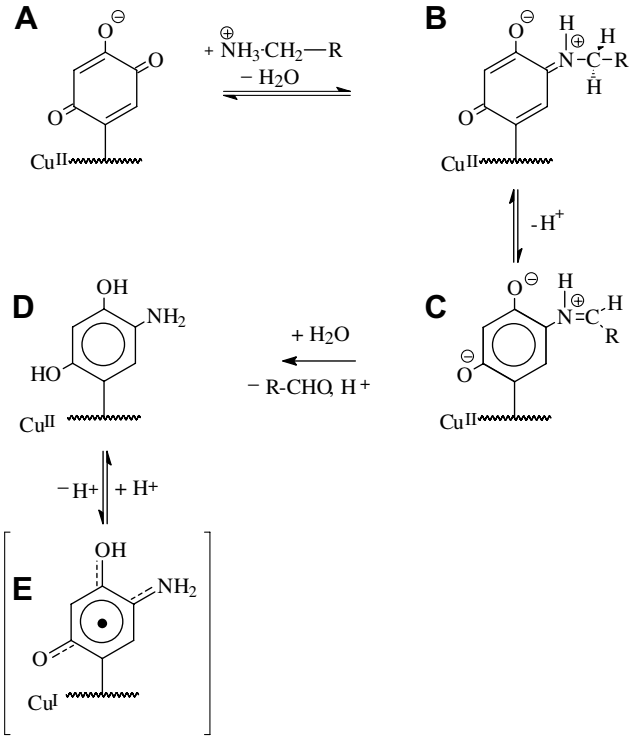

Scheme 1. The reducing half-reaction in the catalytic cycle of pig kidney amine oxidase. (A) Oxidized TPQ. (B) The substrate Schiff base Cu^{II}-quinone ketimine. (C) The product Schiff base Cu^{II}-quinolaldimine. (D) Cu^{II}-aminquinol. (E) Cu^{I}-semiquinolamine radical (TPQ_{sq}). The radical is off the reaction pathway.

the substrate-reduced colorless Cu^{II}-aminoquinol (Scheme 1D; TPQ_{aqu}) equilibrates rapidly with the yellow EPR-detectable Cu^{I}-TPQ_{sq} by transferring one electron to copper ([7] and references therein). While in plants the TPQ_{sq} intermediate could be involved in the amine oxidation mechanism, in the yeast *Hansenula polymorpha* AO there is evidence against the reduction of copper during dioxygen activation [8]. In bovine serum amine oxidase, the TPQ_{sq} species is scarcely populated and reduction of copper is not observed during the conversion of dioxygen into hydrogen peroxide [9]. It is still uncertain whether copper reduction and semiquinone formation are on the catalytic pathway in *Arthrobacter globiformis* AO [10].

The role of copper as cofactor in the catalytic mechanism of AOs has been partially solved by studying copper-free and metal-substituted forms of these enzymes [10–14]. In mammalian intracellular AOs the role of copper has been poorly investigated [15] but the radical species was observed in PKAO, in the presence of cyanide which stabilizes the Cu^{I} form [16].

The present paper describes the properties of Cu-depleted AO from pig kidney. Spectroscopic properties and kinetics of Cu-devoid and Cu-reconstituted enzyme are also reported and compared with AOs from different sources.

2. Materials and methods

2.1. Materials

1,5-Diaminopentane dihydrochloride (cadaverine), benzylamine hydrochloride, phenylhydrazine hydrochloride (PHY) and *p*-(dimethylamino)benzylamine hydrochloride (*p*-DABA) were from Sigma-Aldrich (St. Louis, MO). $CuCl_2$ was from Merck (Darmstadt, Germany). All other chemicals were the purest grade commercially available. PKAO was purified according to a previously described procedure [5]. Protein samples of the highest purity were utilized and showed always a TPQ/dimer ratio 1.9–2.1. The concentration of the quinone content was determined by titration with the carbonyl reagent PHY giving a hydrazone with an extinction coefficient at 442 nm ($\varepsilon_{442} = 5.3 \times 10^4\ M^{-1}\ cm^{-1}$; [5]). The concentration of the purified enzyme ($A_{278}/A_{490} = 75$) was determined using an ε_{490} of 4000 $M^{-1}\ cm^{-1}$ or an ε_{278} of $3 \times 10^5\ M^{-1}\ cm^{-1}$ (two copper ions and a molecular mass of 180 kDa; [5]). For TPQ_{sq} an extinction coefficient of 7100 $M^{-1}\ cm^{-1}$ at 464 nm was used [17]. Protein concentration was also measured by the Lowry method as modified by Hartree [18] with bovine serum albumin as standard. Amine oxidase from lentil seedlings (LSAO) was purified as previously described [19].

2.2. Preparation of Cu-depleted PKAO (Cu_{dep})

All buffers were prepared using distilled water degassed and purged for 3 h with argon gas. Cu_{dep} PKAO was prepared as follows:

Step 1.

To 1 mL PKAO solution (10 μM) 1 mM KCN was added for 1 h, and then dialyzed, under strictly anaerobic conditions, in 100 mM Tris–HCl buffer, pH 7.25, containing 10 mM KCN, at 4 °C for 6 h.

Step 2.

The solution was made 1 mM in *p*-DABA and allowed to stand at 4 °C for 90 min. Afterwards, the solution was dialyzed against 3×500 ml buffer changes (100 mM Tris–HCl, pH 7.25) and then centrifuged at $27\,200 \times g$ for 30 min.

2.3. Spectroscopic methods

Absorption spectra of PKAO in 100 mM Tris–HCl buffer, pH 7.25, were recorded at 25 °C with an Ultrospec 2100 spectrophotometer (Biochrom Ltd., Cambridge, England). Anaerobic experiments were made in a Thunberg-type spectrophotometer cuvette (Soffieria Vetro, Sassari, Italy). Solutions were subjected to several cycles of evacuation followed by flushing with Argon. Anaerobic additions of various reagents to the cuvette were made through a rubber cap with a syringe.

2.4. Determination of metals

Cu, Co, Zn, and Ni content was determined by atomic absorption using a Perkin–Elmer 3030 apparatus. The spectral lines chosen were 324.7 nm for Cu, 340.5 nm for Co, 305.1 nm for Ni, and 202.5 for Zn, respectively.

2.5. PKAO activity

Enzyme activity was measured by oxygen uptake determined with a Clark-type electrode coupled to a OXYG1 Hansatech oxygraph (Hansatech Instruments Ltd. King's Lynn, Norfolk, England). The temperature of reaction chamber was controlled at 35 °C using a circulating water bath. The solution (1 ml) containing the enzyme in 100 mM Tris–HCl buffer, pH 7.25, was maintained for 20 min at constant level of pure oxygen as reported [8,10] and the reaction was started by addition of 17 mM cadaverine. The value of K_M for PKAO using different substrate concentrations (1–5 mM) at saturating concentration of oxygen (219 μM), or varying concentrations of oxygen (5–100 μM) at a saturating concentration of substrate (17 mM cadaverine), was calculated from initial velocity data fitted to the Michaelis–Menten equation by nonlinear regression and by double reciprocal plots by Michaelis–Menten analysis in 100 mM Tris–HCl buffer, pH 7.25. Catalytic-centre activity (k_{cat}) was defined as mol of substrate consumed/mol of active sites $\times\ s^{-1}$.

Benzylamine oxidase activity was measured in 100 mM Tris–HCl buffer, pH 7.25 and 25 °C, by monitoring the increase in absorbance at 250 nm using an $\varepsilon_{250} = 12.8\ mM^{-1}\ cm^{-1}$ for benzaldehyde [17].

Ammonia production was checked from the amount of NADH consumed in the presence of glutamate dehydrogenase. Hydrogen peroxide formation was detected with the peroxidase/4-hydroxy-3-methoxyphenylacetic acid method ([5] and references therein).

All data reported are the mean of five different experiments.

2.6. Determination of TPQ stoichiometry

Native, Cu_{dep} and Cu_{rec} PKAO forms were titrated anaerobically with cadaverine or benzylamine as substrates. PKAOs were also titrated with the carbonyl-reagent PHY.

PKAO was stained after polyacrylamide gel electrophoresis under denaturing conditions (SDS–PAGE) using nitroblue tetrazolium according to the reported procedure for quinoprotein detection [20].

3. Results

3.1. Reaction of pDABA with PKAO

p-DABA is a very poor substrate for PKAO.The k_{cat} value for *p*-DABA in 100 mM Tris–HCl buffer, pH 7.25 and 35 °C, was $3.3(\pm 0.2) \times 10^{-4}$ s^{-1}, a very small value compared with those found for cadaverine (4 ± 0.3 s^{-1}) or benzylamine (0.23 ± 0.01 s^{-1}).

When native PKAO (10 μM) was incubated in aerobiosis with benzylamine (80 μM) a stoichiometry of 1 mole of each product (aldehyde, ammonia, and hydrogen peroxide) per mol of oxygen consumed was observed. Addition of benzylamine or cadaverine to PKAO in aerobiosis induced a bleaching of the 490 nm absorption band (not shown), indicating the rapid conversion of the TPQ cofactor to the bleached TPQ_{aqu} species (Scheme 1D). After the substrate had been exhausted, the typical 490 nm band was restored [5]. When the same experiment was performed under anaerobic conditions and in the presence of 100 μM CN^-, the 490 nm band disappeared immediately and the TPQ_{sq} radical, with absorption peaks at 464, 434 and 360 nm, was observed (Fig. 1; Scheme 1E), as previously reported [5,16]. The ESR spectrum obtained after the anaerobic addition of benzylamine or cadaverine to the cyanide-PKAO complex confirmed the formation of the TPQ_{sq} (not shown) [16]. Instead, when *p*-DABA was added to PKAO in anaerobiosis, the broad absorption band at 490 nm rapidly disappeared indicating the conversion of TPQ to a quinolaldimine. Meanwhile, a new band centered at 410 nm appeared (Fig. 2A). This band was tentatively assigned to the resonance structure quinolaldimine ↔ quino-imine (Fig. 3B) [21,22]. This structure slowly decayed to the product (λ_{max} = 350 nm) identified as *p*-(dimethylamino)benzaldehyde, the well-known Ehrlich's reagent, with a clear isosbestic point at 375 nm (Fig. 2A). 2 ± 0.15 mol aldehyde per enzyme dimer was released at the end of the reaction (4 h). However, when *p*-DABA was added to PKAO in anaerobiosis and in the presence of 100 μM CN^-, the broad absorption band at 490 nm rapidly disappeared and the band centered at 410 nm was formed as in experiments performed without CN^-, but a new broad absorption band and 460 nm appeared (Fig. 2B). Both the 410 and 460 nm bands decayed at similar rate as the 350 nm band grew up. The absence of bands at 464, 434 and 360 nm was indicative for the lack of formation of a free radical intermediate with this substrate. The present results with *p*-DABA were at variance from those obtained for LSAO in which the formation of the quino-imine was followed by its disappearance and the contemporary formation of the aldehyde and the radical species in both the presence and absence of CN^-. In this process isosbestic points at 372, 440 and 478 nm were observed (Fig. 4).

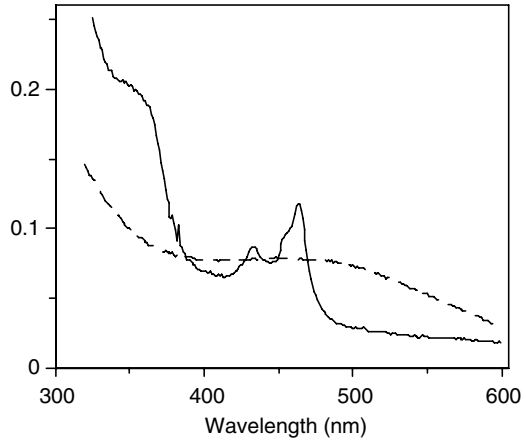

Fig. 1. Absorption spectra of 19 μM PKAO in 100 mM Tris–HCl buffer, pH 7.25. Native enzyme before (----) and after (—) addition of 10 mM cadaverine in anaerobic conditions and in the presence of 100 μM CN^-.

3.2. Properties of Cu-depleted PKAO

The residual metal in Cu_{dep} PKAO, determined by atomic absorption, was shown to be 0.005 ± 0.0002 mol atom mol^{-1} subunit, less than 1% that of the native enzyme. Cu_{dep} PKAO

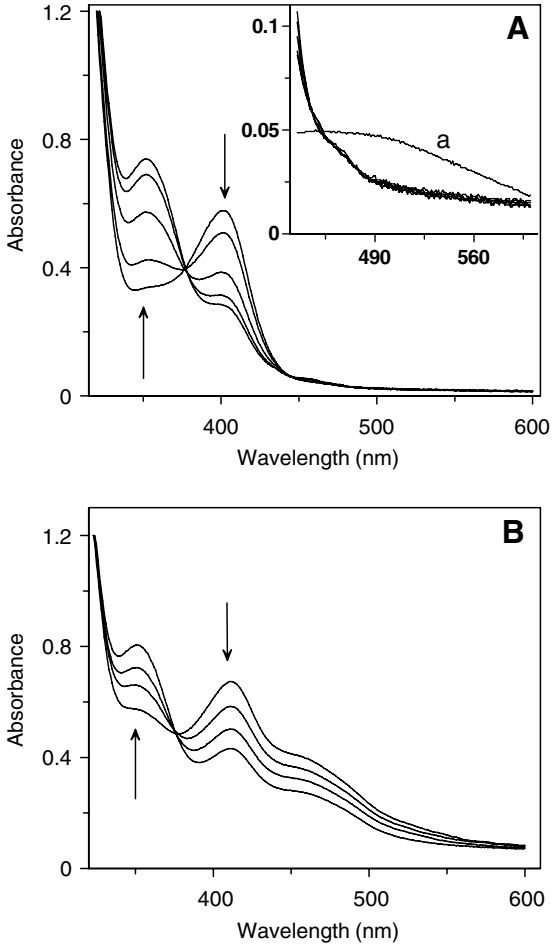

Fig. 2. Panel A. Spectral changes during the reaction of 12 μM PKAO with of 2 mM *p*-DABA, in 100 mM Tris–HCl buffer, pH 7.25, in anaerobic conditions. Spectra were recorded from 120 min to 240 min at intervals of 30 min and show the decay of the 410-nm intermediate (a quino-imine) and the formation of 350 nm band, the *p*-(dimethylamino)benzaldehyde. Inset: 420–600 nm absorption amplified. Trace (a) shows the native enzyme. Panel B. 13.5 μM PKAO in the presence of 100 μM CN^- and 2 mM pDABA. Other experimental conditions as in panel A.

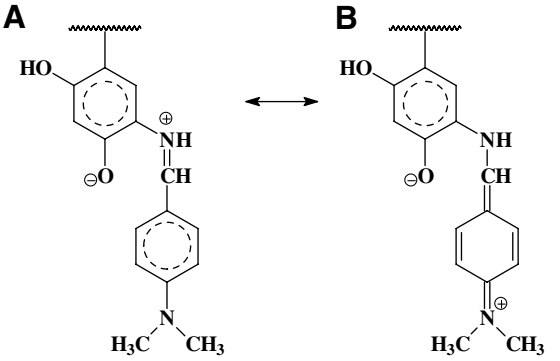

Fig. 3. Reaction of TPQ and *p*-DABA: (A) quinolaldimine; (B) its resonance structure, a quino-imine.

was still pink, showing a broad absorption band at 490 nm, as in the native enzyme. It clearly indicated that TPQ was still in its oxidized form (TPQ_{ox}; Fig. 5). No significant differences in the UV region were seen (not shown).

The addition of benzylamine or cadaverine to Cu_{dep} PKAO in air or in anaerobiosis was accompanied by a rapid bleaching of the visible absorption, as in the native enzyme (Fig. 5), but in this case the 490 absorption band was not restored even after dialysis. TPQ_{sq} (peaks at 464, 434 and a shoulder at 360 nm) was never formed, whereas a sharp absorption at 330 nm appeared and reached its maximum after complete reaction. This band was tentatively identified as aminoquinol (TPQ_{aqu}). The titration of 20 nmol active sites of Cu_{dep} PKAO with benzylamine required about 20 nmol of substrate (not shown), and released 20 ± 2 mol of benzaldehyde as determined by the increase of absorbance at 250 nm.

Cu_{dep} PKAO reacted with PHY up to a stoichiometry of about 2 mol of the carbonyl reagent per mol of dimeric enzyme. The absorption change due to formation of the complex between TPQ and PHY was rather slow, requiring approxi-

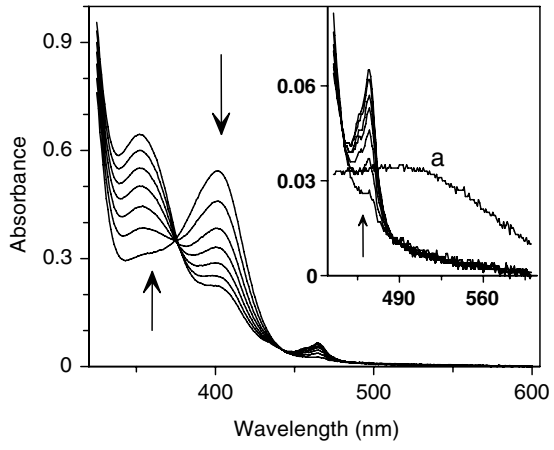

Fig. 4. Spectral changes during the reaction of 9 μM LSAO in the presence of 100 μM CN^- after addition of 10 mM *p*-DABA, in 100 mM Tris–HCl buffer, pH 7.25, in anaerobic conditions. Spectra were recorded from 1 min to 120 min at intervals of 20 min and show the decay of the 400-nm intermediate (a quino-imine) and the contemporary formation of the TPQ_{sq} radical species (464 and 434 nm) and the 350 nm band, the *p*-(dimethylamino)benzaldehyde. Identical spectra are obtained in the absence of CN^-. Inset: Radical intermediate absorption amplified. Trace (a): native enzyme.

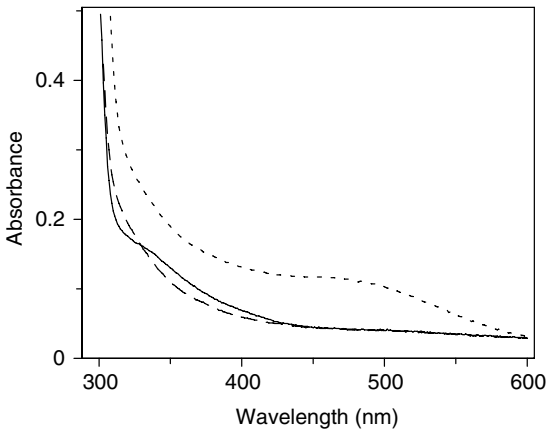

Fig. 5. Absorption spectra of 27 μM copper-free PKAO in 100 mM Tris–HCl buffer, pH 7.25. Dotted line: Cu_{dep} PKAO obtained after treatment with *p*-DABA. Solid line: Cu_{dep} PKAO obtained after treatment with cadaverine or benzylamine (TPQ_{aqu2}). Dashed line: Cu_{dep} PKAO obtained after treatment with dithionite (TPQ_{thb}).

mately 15 min to reach the end point. Instead, TPQ_{aqu} did not react to any extent with PHY.

3.3. Preparation and properties of Cu-fully-reconstituted (Cu_{rec}) PKAO

Copper was reintroduced to the enzyme by incubation of pink Cu_{dep}-TPQ_{ox} PKAO (2 ml) with 2 mM $CuCl_2$ for 3 h followed by dialysis against 5 × 500 ml buffer changes (100 mM Tris–HCl, pH 7.25), for 12 h. The concentration of Cu, taken up by $PKAO_{rec}$ as determined by atomic absorption, was shown to be 1.9 ± 0.03 mol per mol of protein, i.e. almost the full complement of metal found in the native enzyme (Table 1). The K_M values for amines and oxygen of Cu_{rec} PKAO were similar to those found for the native enzyme (0.1 mM for cadaverine and benzylamine and 10 μM for oxygen; Table 2), and the catalytic activity toward cadaverine or benzylamine, at saturated concentrations of amines and oxygen, was about 90–95% that of the native enzyme, in keeping with the amount of copper incorporated. Cu_{rec} PKAO was fully inactivated by PHY with a stoichiometry of about 1/1 (PHY/monomer) much like the native enzyme (Table 1). The reaction was very fast, the time required for maximum spectral change in excess PHY being ≈2 min.

Table 1
Copper content and activity of native and modified pig kidney amine oxidase

Enzyme	k_{cat} (s^{-1})	Metal ion content (%)	PHY/TPQ ratio
Native	4 ± 0.30	100	2.0 ± 0.10
Cu_{dep}[a]	ND	0.5	1.9 ± 0.15
Cu_{dep}[b]	ND	0.5	–
Cu_{dep}[a]	3.5 ± 0.20	90	1.8 ± 0.15
Cu_{dep}[b]	ND	80	–
Co_{sub}	0.1 ± 0.015	90	1.7 ± 0.10

k_{cat} (catalytic-centre activity) is defined as mol of oxygen consumed/mol of active sites × s^{-1}, in 100 mM Tris–HCl buffer, pH 7.25, at saturating concentrations of oxygen and cadaverine. ND, not detectable.

[a]Obtained by treatment with *p*-DABA on copper removal.

[b]Obtained by treatment with sodium dithionite or using cadaverine on copper removal.

Table 2
Kinetics parameters for native an Co-substituted PKAO

Enzyme	k_{cat} (s^{-1})	K_M cadaverine (μM)	k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)	K_M (O_2) (μM)	k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)
Cu-PKAO	4	100	4×10^{-2}	10	0.4
Co-PKAO	0.1	1000	1×10^{-4}	10	0.01

Experimental conditions as in Table 1.
S.D. is omitted.

Electrophoresis under nondenaturing conditions and SDS–PAGE staining for quinoproteins showed the same pattern as the native enzyme (Figure 1, Supplementary data). The ESR spectrum of Cu_{rec} PKAO was quite similar to that of the native enzyme (Figure 2, Supplementary data).

3.4. Irreversible reduction of TPQ

When attempts were made to obtain copper-free enzyme using sodium dithionite or good substrates (cadaverine or benzylamine) to reduce the TPQ cofactor (Step 2), an almost total removal of copper (~99%) was observed together with a reduction of the oxidized TPQ. The TPQ reduction was also obtained after prolonged treatment with *p*-DABA (⩾4 h). We dubbed trihydroxybenzene, TPQ reduced by dithionite (TPQ_{thb}) the TPQ cofactor after dithionite treatment (Fig. 6B) and TPQ_{aqu2} after cadaverine (or benzylamine) treatment (Fig. 6C) (we indicated TPQ_{aqu} the reduced form of TPQ after treatment with *p*-DABA). Following exhaustive dialysis, the TPQ_{aqu2} exhibited an extra broad absorption at around 330 nm (Fig. 5) that was not present in TPQ_{thb} (Fig. 5), confirming that the 330 nm band was due to the aminoquinol form. We failed to obtain active PKAO by incubating Cu_{dep}-TPQ_{thb} or Cu_{dep}-TPQ_{aqu2} with 2 mM $CuCl_2$ for 3–24 h. Although the final concentration of copper was about 1.8 ± 0.02 mol per mole of reconstituted protein (Table 1) and nitroblue tetrazolium staining for quinoproteins was only slightly fainter (See Figure 1, Supplementary data), the enzyme stayed irreversibly reduced. Neither TPQ_{thb} nor TPQ_{aqu2} reduced forms did react any more with PHY (up to 48 h; Table 1) confirming that the enzyme, under these conditions, was irreversibly reduced. No indications of gross conformational changes in Cu_{dep}-TPQ_{aqu2} were sought by near or far UV CD, intrinsic fluorescence or 1-anilinonaphthalene 8-sulfonate (ANS) binding fluorescence (not shown).

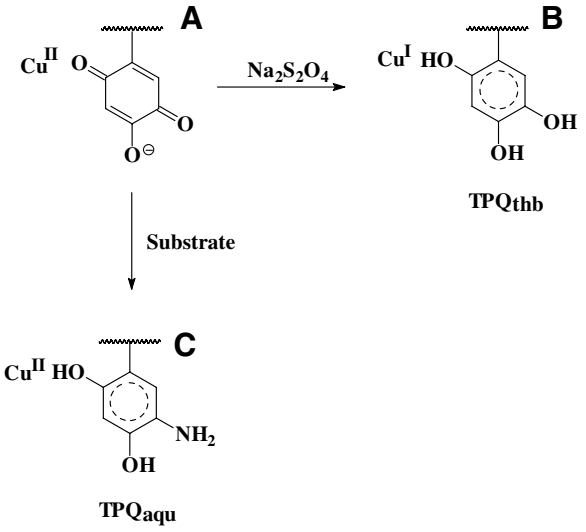

Fig. 6. Scheme showing the reduction of TPQ_{ox} to TPQ_{red} with sodium dithionite or with a substrate. (A) TPQ_{ox}; (B) trihydroxybenzene (TPQ_{thb}); (C) aminoquinol (TPQ_{aqu}; we indicated TPQ_{aqu} the reduced form of TPQ after treatment with *p*-DABA, and TPQ_{aqu2} after cadaverine (or benzylamine) treatment).

The copper/TPQ AOs share the ESR parameters of the so-called type 2 Cu-ESR spectra [22,23]. Native PKAO, as well as fully active Cu_{rec} enzyme showed an ESR spectrum characterized by $A_{\parallel} = 151$ G , $g_{\parallel} = 2.280$, and $g_{\perp} = 2.051$. The Cu_{rec}-TPQ_{aqu2} form of the enzyme showed subtle but significant changes of the typical type 2 copper spectrum parameters, having $A_{\parallel} = 150$ G, $g_{\parallel} = 2.295$, and $g_{\perp} = 2.065$, indicative of an inner sphere perturbation of the coordination to the copper [5].

As reported above, the addition of a substrate to Cu_{dep}-TPQ_{ox} PKAO was accompanied by a rapid bleaching of the visible absorption and by the formation of a sharp band at 330 nm (TPQ_{aqu}). Addition of $CuCl_2$ to TPQ_{aqu} did not restore the oxidized TPQ indicating again an irreversible reduction of TPQ.

3.5. Copper cannot be substituted

Co-, Ni- and Zn-substituted PKAOs were tentatively prepared by incubation of Cu_{dep} PKAO with 2 mM $NiCl_2$, $ZnCl_2$ or $CoCl_2$, respectively, for 3 h followed by dialysis against 5 × 500 ml buffer changes (100 mM Tris–HCl, pH 7.25), for 12 h, as reported for the preparation of Cu_{rec} PKAO. After dialysis, Ni and Zn were not bound in Cu-depleted PKAO. Instead, nearly stoichiometric incorporation of Co in the protein (1.7 ± 0.2 mol per mole; Table 1) was observed by atomic absorption spectrometry. The Co substituted PKAO was active though at lower extent (Table 1). Its K_M value for cadaverine was considerably higher to those of native or Cu_{rec} enzyme (Table 2), whereas K_M values for oxygen was similar (Table 2). Further incubation with $CoCl_2$ neither changed the Co content, nor the activity. The reactivity with PHY of Co-PKAO was lower that of Cu-native enzyme, the absorption change due to formation of the complex between TPQ and PHY requiring approximately 6 min to reach the end point, even though it was significantly more reactive than Cu_{dep}-PKAO.

The addition of excess cadaverine to Co-substituted PKAO in air was accompanied by a rapid bleaching of the visible absorption at 490 nm that was restored only after 60 min, in line with the lower enzyme activity (not shown). Under anaerobic conditions, a rapid bleaching of the 490 nm absorption and the formation of a band at 330 nm, indicative of the formation of TPQ_{aqu}, were observed (Fig. 5). No peaks at 464, 434 and 360 nm were apparent during the process, seemingly ruling out the formation of a free radical intermediate in Co-substituted enzyme.

4. Discussion

The role of copper in the catalytic mechanism of AOs has been a controversial matter for about 40 years ([6] and references therein). All Cu/TPQ AOs contain a copper ion in a 1:1 stoichiometric ratio with the TPQ cofactor. X-ray crystallography of AOs from bacteria, yeasts, plants and mammals shows that the copper ion is coordinated to three hystidine residues and two water molecules, and is located very close, but not bound, to the TPQ cofactor in the so called "*off copper*" conformation [24].

In all the studied AOs the reductive half-reaction, leading from the oxidized TPQ to the formation of the aminoquinol, proceeds independently of the copper ion, demonstrated by the ability of copper-free LSAO to oxidize amines to the corresponding aldehydes [25]. The more debated oxidative half-reaction, from the TPQ_{aqu} to the TPQ_{ox}, involves a two proton and two electron transfer from aminoquinol to dioxygen where the copper ion appears to play a key role. This role has been partially clarified by studying copper-free or copper-substituted enzymes. These experiments have been done with AOs from the bacterium *Arthrobacter globiformis* [10], from the yeast *H. polymorpha* [13], from plants [14], and from bovine plasma [6,11,12]. While the evidence from plant amine oxidases supports transient reduction of copper being involved in the oxidative half-reaction, with TPQ_{sq} being the species that preferentially reacts with dioxygen, in bovine serum and *H. polymorpha* AOs, it has been demonstrated that dioxygen reduction does not require copper reduction, and the formation of the radical species if off the reaction pathway. The copper ion has an essential role in catalyzing the electron transfer between the reduced TPQ and dioxygen in *Arthrobacter globiformis* AO, but the authors were not able to find the TPQ_{sq} on the reaction pathway [10].

In this paper, we add further details to the picture by obtaining a Cu_{dep} AO from pig kidney through a new procedure utilizing the poor substrate *p*-DABA. Copper-free PKAO, containing less than 1% of the copper complement, is still pink with a broad absorption peak in the visible region at 490 nm like the native enzyme, confirming that TPQ, in its oxidized form, is responsible for this absorption. The Cu_{dep} form is still able to oxidize one equivalent of substrate/subunit under single turnover conditions further indicating that copper is not essential for the reductive half-reaction in AOs. Moreover, this apo-enzyme reacts with phenylhydrazine with the same stoichiometry as the native enzyme, indicating a fully reactive TPQ.

When reduced by a substrate, Cu_{dep}-TPQ_{aqu} shows an absorption at 330 nm. Although the aminoquinol form of TPQ observed in copper/TPQ AOs from bovine serum [21] and *H. polymorpha* [26], and in model compounds [27], shows an absorption around 310 nm, we believe that also the 330 nm absorbance is due to the aminoquinol. As a matter of fact it is not observed in dithionite-reduced TPQ where no amino group is present.

Reduction of the enzyme by sodium dithionite or by the "good" substrate cadaverine in the presence of cyanide (Step 2) results, much like as seen with *p*-DABA, in a >99% copper removal. In this case, however, the TPQ cofactor stays reduced (TPQ_{aqu2}) and resistant to reoxidation by air, as reported in other AOs [8,10,13]. Surprisingly, after addition of copper ions to the Cu_{dep}-TPQ_{aqu2} protein followed by exhaustive dialysis, although the copper content is back up to >80% to that of the native enzyme and the nitroblue tetrazolium staining is consistent with a redox active TPQ, the enzyme is unable to react with substrates or with PHY. Fluorescence and CD data demonstrate that tertiary and secondary structures of the protein are not grossly modified, whereas ESR measurements show a small perturbation of the copper coordination, indicating a more axial geometry. These results can be tentatively interpreted as follows:

(i) PKAO, in the presence of *p*-DABA, forms the substrate Schiff base quinoketimine that proceeds rapidly to the product Schiff base quinolaldimine, which is in equilibrium with its resonance structure, the quino-imine. This process makes the quinolaldimine relatively stable to hydrolysis since the formation of TPQ_{aqu} with the simultaneous release of the aldehyde product only started after a lag-phase of 120 min. Thus, in strictly anaerobic conditions and in the presence of CN^- there may be a back reaction from quinolaldimine to quinoketimine, leaving TPQ in its oxidized form after the loss of copper. This hypothesis is confirmed by the observation that a longer incubation with *p*-DABA, i.e. long enough to form the aminoquinol, yields a redox inert apoenzyme upon removal of copper by CN^-.

(ii) In the presence of a good substrate, the rapid release of the aldehyde product and the formation of the radical species prevented the above reported back reaction. TPQ, having a certain degree of mobility, might rotate pointing the oxygen in position 4′ toward the copper site. Thus TPQ might become firmly coordinated to copper in the so-called "on copper conformation" [24,28,29]. Since the X-ray structure of PKAO is not yet known, our interpretation is largely hypothetical.

In any case from the above results we can conclude that copper, besides being involved for facilitating transfer of electrons to the oxygen in the reaction catalysed by PKAO, might be also required for maintaining the active site geometry. The precise geometry around the active site must be crucial because several AOs from other sources are not inactivated after copper removal by dithionite. It is indeed worth recalling that fully-active enzymes can be recovered from copper-free AOs after the treatment with dithionite in *Arthrobacter globiformis* [10] and in bovine serum [12], whereas dithionite irreversibly damages the AO from *H. polymorpha* [8].

The addition of copper ions to Cu_{dep}-TPQ_{ox} PKAO restores catalytic activity up to 90% with kinetic parameters very similar to those of the native enzyme. Cobalt can partly mimic the role of copper as reported in other proteins [11–13], and Co-substituted PKAO shows the ability of oxidizing the substrate, although at lower rate, and to react with PHY. We can exclude that the low activity observed in Co-substituted enzyme can be accounted for by residual copper. In contrast, neither Ni^{II} nor Zn^{II} appear to bind or reactivate the Cu_{dep} and, due to the lack of information about PKAO structure, we are unable to propose a structural mechanism to explain the differences of interaction between the metal ions.

Another interesting result is that PKAO forms TPQ_{sq} in anaerobiosis and in the presence of CN^- with cadaverine of benzylamine but not with *p*-DABA. We do not believe from our results that this reflects the lower catalytic activity toward the latter substrate. In fact LSAO always forms the semiqui-

nolamine radical either with putrescine ($k_{cat} = 150\ s^{-1}$), benzylamine ($k_{cat} = 0.6\ s^{-1}$) or *p*-DABA ($k_{cat} = 2.3 \times 10^{-4}\ s^{-1}$), whereas PKAO forms the semiquinolamine radical with cadaverine ($k_{cat} = 4\ s^{-1}$) and benzylamine ($k_{cat} = 0.23\ s^{-1}$), but not with *p*-DABA ($k_{cat} = 3.3 \times 10^{-4}\ s^{-1}$). Moreover, the results obtained with *p*-DABA do not provide evidence of a copper reduction on the reaction pathway for PKAO. Another possibility for not observing the radical species and copper reduction in the presence of *p*-DABA is that the quinolaldimine, in equilibrium with quino-imine intermediate, is relatively stable to hydrolysis leading to a very low steady-state concentration of TPQ_{aqu}. This could also in part explain why the catalytic activity is so much slower in the presence of *p*-DABA as substrate. Finally the 460 nm band observed during the reaction of PKAO with pDABA in the presence of CN^-, but not observed in LSAO, may be interpreted as a different active site in the two enzymes.

All the results obtained in the present work for the oxidative half-reaction of PKAO can be interpreted with the direct reduction of dioxygen by aminoquinol not involving a change in the redox state of copper. Moreover, the semiquinone radical is not formed and, therefore, PKAO would be numbered with AOs in which the semiquinone is not on the reaction pathway, like the enzymes from *H. polymorpha* and from bovine plasma. It is possible, but not probable, that TPQ_{sq} is always formed but at variable steady-state concentrations depending on the redox potential of the couple Cu-TPQ and on the geometry of the active site. As a matter of fact neither Zn^{II} nor Ni^{II} can substitute copper in the active site of PKAO and Co^{II}, which instead binds, it is only able to support a tiny fraction of activity. Thus the precise nature of the metal is very critical for enzymatic turnover. The differences in K_M for the amine substrate between the two metal-forms of PKAO suggest that copper can play an important role in orienting the TPQ ring correctly.

In conclusion, a further comparative investigation of the structure and function of active sites in different AOs is needed to understand whether these enzymes were working with an overall similar mechanism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.06.089.

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